Molecular Ecology Notes (2007) 7, 937–946 doi: 10.1111/j.1471-8286.2007.01804.x

TECHNICAL ARTICLE

Highly accurate SNP genotyping from historical and low-quality samples

PHILLIP A. MORIN and MELISSA MCCARTHY*
Southwest Fisheries Science Center, 8604 La Jolla Shores Drive, La Jolla, CA 92037, USA

Abstract

Historical and other poor-quality samples are often necessary for population genetics, conservation, and forensics studies. Although there is a long history of using mtDNA from such samples, obtaining and genotyping nuclear loci have been considered difficult and error-prone at best, and impossible at worst. The primary issues are the amount of nuclear DNA available for genotyping, and the degradation of the DNA into small fragments. Single nucleotide polymorphisms offer potential advantages for assaying nuclear variation in historical and poor-quality samples, because the amplified fragments can be very small, varying little or not at all in size between alleles, and can be amplified efficiently by polymerase chain reaction (PCR). We present a method for highly multiplexed PCR of SNP loci, followed by dual-fluorescence genotyping that is very effective for genotyping poor-quality samples, and can potentially use very little template DNA, regardless of the number of loci to be genotyped. We genotyped 19 SNP loci from DNA extracted from modern and historical bowhead whale tissue, bone and baleen samples. The PCR failure rate was < 1.5%, and the genotyping error rate was 0.1% when DNA samples contained > 10 copies/μL of a 51-bp nuclear sequence. Among samples with ≤ 10 copies/μL DNA, samples could still be genotyped confidently with appropriate levels of replication from independent multiplex PCRs.

Keywords: ancient DNA, bowhead whale, population genetics, single nucleotide polymorphism, SNP

Received 24 January 2007; revision accepted 20 March 2007

Introduction

Single-nucleotide polymorphism (SNP) genotyping is rapidly becoming a powerful tool for assessing genetic variation in natural populations (reviewed in Brumfield *et al*. 2003; Morin *et al*. 2004). Recent applications show that SNPs are extremely frequent and relatively easy to ascertain in many nonmodel organism genomes (Primmer *et al*. 2002; Aitken *et al*. 2004; Elfstrom *et al*. 2006; Morin *et al*. 2007a), and can be applied to a wide range of population studies, from individual identification to population structure and taxonomy (Kuhner *et al*. 2000; Glaubitz *et al*. 2003; Smith *et al*. 2004; Seddon *et al*. 2005; Elfstrom *et al*. 2006). Some of the benefits of using SNPs relative to other nuclear markers such as microsatellites include ease and efficiency of discovery and genotyping (e.g. Elfstrom *et al*. 2006; Morin *et al*. 2007a), ability to target variation in random genomic regions or known genes (Aitken *et al*. 2004; Kohn *et al*. 2006), and existence of theoretical treatment (Chakraborty *et al*. 1999; Nielsen 2000; Kalinowski 2002; Hedrick 2005; Ryman *et al*. 2006) and analysis tools and methods for assessing power and population parameters (Ryman 2006; Ryman & Palm 2006).

Although the potential application of SNPs to degraded, historical, and ancient samples has been discussed in the literature (Surridge *et al*. 2002; Noonan *et al*. 2005; Asher & Hofreiter 2006; Poinar *et al*. 2006; Römpler *et al*. 2006b), there are few examples to date (Römpler *et al*. 2006b). As for all degraded samples, the issues of working with few copies of DNA pertain to SNPs: contamination, allelic dropout, and polymerase chain reaction (PCR) failure.

Correspondence: P. A. Morin, Fax: 858-546-7003; E-mail: phillip.morin@noaa.gov
*Present address: Stratagene, 11011 N. Torrey Pines Road, La Jolla, CA 92037, USA

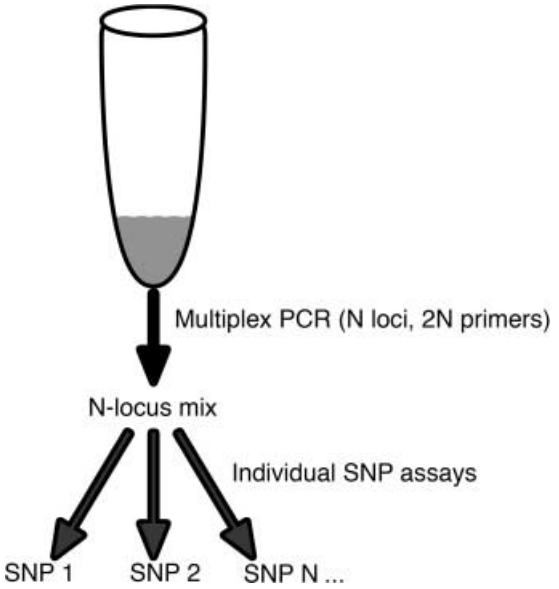

Fig. 1 Schematic diagram of the multiplex PCR of all loci, followed by genotyping of individual SNPs with Amplifluor assays. Replication of PCR for genotype verification occurs at the multiplex PCR step, followed by genotyping of all assays from each multiplex PCR product.

Ways to minimize the former have been described in detail (Pääbo *et al*. 2004; references therein). For addressing potentially high rates of PCR failure and allelic dropout, several approaches have been proposed, but most require replicate PCR to ensure that both alleles are detected (Taberlet *et al*. 1996; Morin *et al*. 2001). This replication can be particularly troublesome when the number of loci to be genotyped is high, and amount of starting material is low, as DNA may be used up long before all loci are genotyped and replicated sufficiently. For SNPs to be useful for these types of studies, they need to be assayed from relatively small DNA fragments (< 150 bp), and to make use of multiplex PCR to minimize the number of PCRs performed from each extract. Recent advances in multiplex PCR methodologies now appear to make screening of nuclear genetic variation possible for the first time from a wide range of poor-quality samples (Lindblad-Toh *et al*. 2000; Markoulatos *et al*. 2002, 2003; Shapero *et al*. 2004; Krause *et al*. 2006; Römpler *et al*. 2006a).

We have previously shown that the Amplifluor SNP genotyping system (Chemicon Inc., Temecula, CA, USA) can be applied effectively to genotyping cetacean SNPs (Morin *et al*. 2007a), but that the assay quality is sensitive to DNA concentration. To overcome this problem we used flanking PCR primers to preamplify individual loci prior to genotyping with the Amplifluor assay. Here, we have further developed this process to allow genotyping of historical and low-quality samples by developing multiplex preamplification of all SNP loci in one PCR prior to performing individual genotyping assays. The use of multiplex preamplification serves several purposes. First, it significantly improves the data quality (signal to noise ratio and separation of allele clusters) and completeness from each Amplifluor assay, allowing easy semiautomated genotype assignment. Second, it reduces the number of times each DNA extract needs to be accessed and used for PCR, as a sample only has to be multiplex PCR amplified once to generate products to be used in all of the SNP assays. Finally, the method described here uses one primer in common with the Amplifluor genotyping assay and a second primer that overlaps the allele-specific amplifluor primers in most cases, to generate very small multiplex products (< 120 bp) for all loci, thereby allowing us to genotype very degraded and low-concentration samples, such as DNA extracted from historical and even ancient samples. A schematic diagram of the whole process is shown in Fig. 1, with replication, where needed for low-quality samples, occurring at the multiplex amplification stage, followed by SNP genotyping with individual assays from each multiplex product.

Materials and methods

DNA extraction and quantification

DNA was extracted from bowhead whale (*Balaena mysticetus*) tissue samples using either silica-based extraction columns (DNeasy, QIAGEN Inc.; X-tractor Gene, Corbette Robotics; Puregene, Gentra Systems Inc.,) or standard phenol–chloroform methods. DNA was extracted from approximately 100–200 mg of historical bone and baleen samples and quantified for nuclear fragment concentration using quantitative PCR (qPCR) of a 51-bp single copy nuclear DNA fragment as described in Morin *et al*. (2007b, 2006). Bone and baleen samples ranged in age from approximately 2–40 years, and were collected from skulls on St. Lawrence Island (SLI), Alaska, or from collections of aboriginal hunters on SLI. The skulls had been weathering on the beach since the whales were killed, and baleen was typically kept in ambient conditions in the homes of the aboriginal hunters.

Assay design

SNP genotyping assays were designed for the Amplifluor genotyping system (Chemicon Inc.). The Internet-based program Amplifluor AssayArchitect™(Chemicon Inc.) was used in Express mode to select two allele-specific forward PCR primers and a common reverse primer, and to add the Amplifluor complementary oligonucleotide tail sequences to the primers. The Internet-based primer selection program PRIMER 3 (Rozen & Skaletsky 2000) was used to select a multiplex forward primer complementary to the Amplifluor reverse primer, and which produced a product

Table 1 List of loci genotyped, SNP type, minor allele frequencies, multiplex product and Amplifluor assay product lengths (bp), and Amplifluor assay conditions: T_a, annealing temperature

Assay*	SNP type	Minor allele frequency (%)	Multiplex product length	Amplifluor product length†	Magnesium concentration (mM)	T_a	No. of cycles	20x primer mix§, comments
Bmys1R248	A/G	0.4	78	60–63	1.5	58	40	
Bmys28R162	A/G	39.8	67	49–51	1.5	58	40	
Bmys31Y94	C/T	13.5	84	73–76	1.8	58	40	
Bmys34M251	A/C	4.0	55	36–38	1.5	58	50	
Bmys42aK46	G/T	47.5	61	51–52	1.8	58	40	1.0 μM T-allele primer
Bmys43Y377	C/T	17.2	86	69–71	1.5	58	40	
Bmys48S269	C/G	42.7	94	73	1.8	58	40	
Bmys60Y260	A/G	3.3	48	41–43	1.5	62	40	
Bmys92K271	G/T	17.1	58	52–54	2.5	58	40	
Bmys96R421	A/G	19.2	85	83	2.5	58	40	
Bmys108D91	C/–	18.6	117–118	97–98	2.5	58	40	SNP is an indel (deletion of a C), assayed as C/G
Bmys368R272	A/G	12.6	81	63–66	1.5	62	40	
Bmys382Y279	C/T	10.6	70	65–66	2.5	58	40	
Bmys387R245	A/G	20.3	90	75–76	1.8	58	40	
Bmys395Y158	C/T	39.4	86	56–57	2.5	62	40	
Bmys396R109	A/G	13.3‡	53	48–49				Could not optimize to resolve alleles
Bmys402M56	A/C	36.5	113	90	1.5	58	50	0.25 μM A-allele primer
Bmys410K107	G/T	41.4	61	52–53	2.5	58	40	
Bmys412S79	A/G	20.6	53	48–49	1.0	60	40	
Bmys414R127	A/G	39.0	69	55–57	2.5	58	40	

*Primer sequences are available from P.A.M. on request.
†Not including amplifluor-specific primer tails; products vary depending on differences in length of allele-specific primers.
‡Based on sequences ($N = 15$).
§Unless otherwise stated, the 20× primer mix for each amplifluor assay contained 0.5 μM of each allele-specific primer, and 7.5 μM common reverse primer.

at least 2 bp larger than the Amplifluor product (excluding the added Amplifluor tail sequences), and did not overlap the SNP site. The melting temperature default target of 60 °C was used for multiplex forward primer design. All assays were designed to amplify products less than 120 bp in length to maximize the chance that they would work on highly degraded samples (see Table 1 for multiplex and Amplifluor assay product sizes).

Multiplex PCR

Multiplex PCRs contained the common reverse primer and external forward primer for each of 20 loci (40 primers). PCR conditions were based on Römpler *et al*. (2006a). Each multiplex PCR for bone and baleen samples was performed in 25 μL total volume, with 5 μL of DNA extract (out of 100 μL total per extract), 1× QIAGEN HotStarTaq PCR buffer, 1 mg/mL BSA, 4.5 mM $MgCl_2$, 250 μM of each dNTP, 0.15 μM of each primer, and 0.05 U/μL HotStarTaq polymerase (QIAGEN). Extraction no-template controls (NTC, carried out at the time of the DNA extractions, with all reagents and steps except the addition of sample) were interspersed approximately every sixth sample to control for cross-contamination of extracts, and to subsequently control for cross-contamination of multiplex PCR products in the genotyping assays. No-template PCR controls were also included to ensure that PCR reagents were not contaminated.

Multiplex PCRs for modern tissue DNA extracts had the same reaction conditions, but were done in a final volume of 20 μL, and contained only 2 μL of DNA extract. PCR cycling conditions included an initial denaturation step at 95 °C for 10 min, followed by 30 cycles at 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, and a final elongation step at 72 °C for 4 min.

Multiplex PCR products were visualized in 2% agarose gels and subsequently diluted 1 : 50 or 1 : 100 in AE buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0; QIAGEN) prior to use for SNP genotyping.

SNP genotyping

Amplifluor genotyping requires five primers. There are three locus-specific unlabelled primers, including two allele-specific forward primers, each with a unique 'tail'

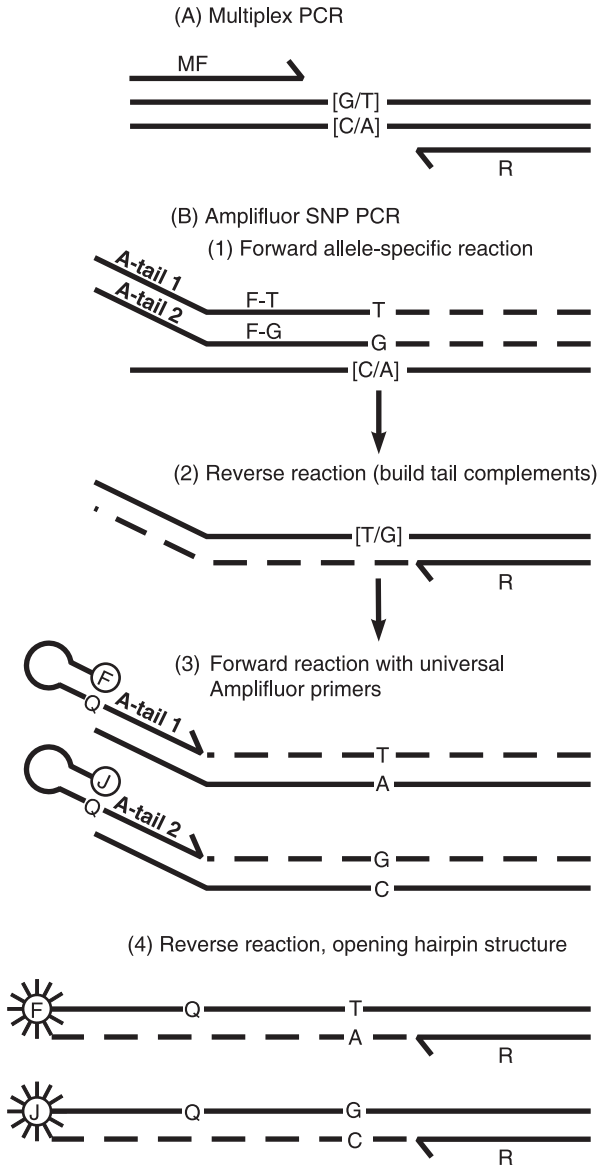

Fig. 2 Amplifluor SNP genotyping process for a G/T SNP. Primers are: multiplex forward (MF), common reverse (R), allele-specific forward primers for the T allele (F-T) and G allele (F–G), and Amplifluor universal primers. The tail sequences on the 5′ ends of the allele-specific forward primers and at the 3′ end of the Amplifluor universal primers are labelled A-tail 1 and A-tail 2. Dabsyl quencher is represented by Q. Fluorophores FAM and JOE are represented by F and J. See text for assay description. Figure modified by permission from Millipore Inc.

sequence on the 5′ end and the 3′ or penultimate 3′ nucleotide complementary to one of the SNP nucleotides (Fig. 2) (for examples, see Morin *et al*. 2007a). These are paired with a common reverse primer which is the same as was used in the multiplex PCR for that locus. After the first extension by the forward allele-specific primers, the reverse primer extends through the end of the forward primer and tail sequences, creating the complement of each tail. Two universal amplifluor primers each have a unique 3′ sequence that is the same as the tail 1 or tail 2 sequence, and a 3′ sequence that forms a stable hairpin loop in solution, and which contains a Dabsyl quencher and fluorophore (FAM or JOE) moiety that are in close proximity while the primer is folded back on itself (Fig. 2). After initial cycles incorporating the allele-specific primer sequence(s) and tail(s), the product can be amplified by the combination of the appropriate Amplifluor primer(s) and the common reverse primer. As the product is replicated the Amplifluor hairpin loops become linearized, separating the fluorophore from the Dabsyl quencher and causing an increase in fluorescence. Allele-specific amplification results in an increase of FAM and/or JOE fluorescent signal for each genotype.

Amplifluor genotyping was performed for each assay individually, using the diluted multiplex PCR product as the template. To facilitate genotyping efficiency and to minimize sample handling steps that could lead to sample mix-ups and cross-contamination, we made master DNA plates containing the diluted multiplex PCR products, extraction control multiplex products, and no-template controls, along with multiplex PCR products of assay control samples (seven samples with known sequences for each locus), and then made replicate plates of these samples with 2 μL of each sample or control. These plates were dried in an incubator at 55 °C for 2–15 h, then sealed with plate-sealing film and stored at room temperature until used (0–30 days) (Morin *et al*. 1999).

Amplifluor assay master mixes were prepared for each dried sample plate, for a final volume of 10 μL per reaction. PCR assay reactions contained 1× Amplifluor assay buffer (Chemicon) with the appropriate $MgCl_2$ concentration (Table 1), 200 μM of each dNTP, 25 nM of each allele-specific forward primer, 375 nM of common reverse primer, 0.5× of each Amplifluor primer (with FAM or JOE fluorescent labels) (Chemicon), and 0.1 μL of Titanium Taq (units unknown; CloneTech Laboratories). PCR cycling and data collection were performed in a Stratagene MX3000P real-time PCR instrument. Cycling parameters included an initial denaturation step at 96 °C for 4 min, followed by 40 or 50 cycles (Table 1) at 96 °C for 10 s, appropriate annealing temperature (Table 1) for 20 s, and 72 °C for 40 s.

Samples were replicated in several ways to verify genotypes and estimate error rates for both parts of the genotyping process: multiplex PCR and Amplifluor genotyping. One sample from high-quality DNA was duplicated on a plate for all assays, replicating both multiplex PCR (2×) and Amplifluor genotyping (19 assays) from a high-quality DNA sample. We replicated genotypes for 85 samples from two to 43 times from the same multiplex PCR product, to estimate the error rates due to amplifluor genotyping methods from multiplex PCR products. To estimate the error rates due to

Table 2 Historical sample data summary. DNA concentration was measured using a single copy nuclear DNA qPCR assay (see Methods). Only samples with ≤ 10 copies/μL DNA concentration were genotyped three to four times to allow determination of the frequency of mismatched genotypes (allelic dropout)

Sample ID	Sample type	qPCR DNA concentration (copies/μL)	Amplifluor PCRs	No. of failures	Failure rate (%)	genotype mismatches
44618	Skull	0	62	3	4.8	0
44636	Baleen	0	61	3	4.9	0
44627	Skull	0	73	3	4.1	1
44638	Baleen	1	73	7	9.6	1
44611	Skull	1	59	13	22.0	5
44621	Skull	2	74	8	10.8	3
44637	Baleen	2	73	5	6.8	1
44605	Skull	2	58	3	5.2	1
44599	Skull	3	58		0.0	1
44612	Skull	4	58	1	1.7	2
44610	Skull	4	58	3	5.2	3
44617	Skull	5	72	16	22.2	3
44602	Skull	5	58	3	5.2	2
44641	Baleen	10	72	3	4.2	1
44626	Skull	11	20		0.0	NA
44604	Skull	15	20		0.0	NA
44603	Skull	17	19		0.0	NA
44642	Baleen	21	20		0.0	NA
44622	Skull	21	21	2	9.5	NA
44623	Skull	24	21		0.0	NA
44608	Skull	91	20		0.0	NA
44609	Skull	225	20		0.0	NA
44606	Skull	256	20		0.0	NA
44600	Skull	320	20		0.0	NA
44607	Skull	958	20		0.0	NA
44624	Skull	1337	20		0.0	NA

NA, not applicable.

multiplex PCR amplification, two methods were used: (i) 15 historical samples were extracted twice and genotypes generated from both replicates, and (ii) 15 samples with ≤ 10 copies/μL (based on qPCR analysis, Table 2) were multiplex amplified three to four times and genotyped from each multiplex PCR to estimate allelic dropout rates and to verify genotypes for these low-quality samples. Genotypes were accepted as 'true' for the purposes of the latter analysis if both alleles of heterozygote genotypes were seen at least twice, and if homozygous genotypes were replicated at least three times. This does not guarantee that these genotypes are in fact correct, but provides a significant increase in probability that they are correct compared to a single genotyping event (Taberlet *et al*. 1996).

Data analysis

Some Amplifluor assays can produce background fluorescence in the absence of PCR product, so it is important to analyse the data at a cycle that maximizes the signal to noise ratio, when background signal of the NTCs is close to zero. Although assays can be genotyped from 'endpoint' fluorescent values, in which PCR is performed in a standard PCR machine and followed by fluorescent detection, this requires careful optimization of the assays to determine the optimal number of cycles. We chose to use real-time detection of fluorescence followed by analysis of the data at the cycle that maximized the signal to noise ratio. This was typically done by viewing the amplification plots for an assay, and setting the cycle for analysis before the NTCs exhibited a significant increase in fluorescence. This allowed us to use the same number of PCR cycles for most assays (Table 1), with the cycle number for analysis chosen post-PCR.

Fluorescence values (dR Last) for each of the allele-specific Amplifluors were plotted for the selected final cycle, and clusters of genotypes and NTCs selected to assign genotypes.

Relative efficiency of PCR for individual loci in the multiplex PCR (e.g. low product yield because of primer–primer interactions) was calculated from the average FAM

or JOE Ct values from amplifluor assays using the multiplex products of seven control samples as template. Ct values are the individual cycle numbers for each sample inferred at the point where fluorescence reaches an arbitrary threshold during PCR amplification. Ct values are inversely proportional to the starting DNA concentration when assay efficiency is equal; when DNA concentrations are constant, Ct values reflect the relative efficiencies of each assay. The ratio of the average Ct values for each assay to the assay with the lowest Ct value (Bmys368, highest inferred efficiency) provides an indication of the relative amount of PCR inhibition for each assay in the multiplex PCR. We have also used the individual pairs of primers for each locus in the multiplex mix to amplify the individual loci from the multiplex product in the presence of Sybr Green double-strand DNA fluorescent stain as another method of estimating the relative quantity of each locus product in the multiplex product (data not shown).

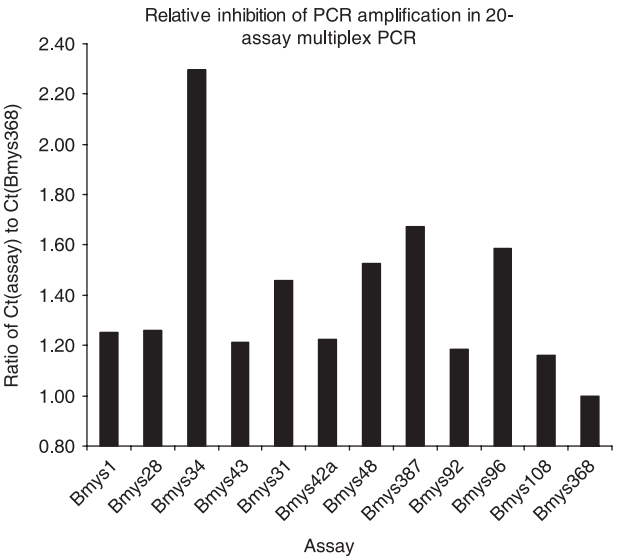

Fig. 3 Relative average inhibition of PCR for 12 assays amplified simultaneously in a 20-assay multiplex PCR (see Methods).

Results

We were able to design 19 of 20 assays automatically using the Internet-based software as described. The software could not select primers for one locus (Bmys96R421), so primers were selected manually. Although the assay Bmys396R109 was ultimately dropped because alleles could not be reliably resolved, multiplex preamplification primers for all 20 assays were included in the primer mix for all of the genotyping described here. Prior to testing multiplex amplification, most of the assays were optimized using genomic DNA from control samples for which we had DNA sequence to validate the genotypes (Table 1). This allowed us to then check the multiplex products for control genotypes to verify that all loci could be genotyped, and to estimate the relative efficiency of each assay in the multiplex PCR. From 12 assays genotyped for seven controls in the same real-time PCR, we found that, although most assays performed similarly, a few assays (e.g. Bmys34M251) have relatively low efficiency (Fig. 3), and could benefit from primer re-design prior to multiplex amplification of samples for subsequent genotyping (H. Römpler, pers. comm.). In our set of 20 loci, two exhibited PCR inhibition (or low efficiency) in the multiplex PCR, but using 50 cycles for the Amplifluor assays was sufficient to compensate for this inhibition.

One locus was virtually monomorphic (one heterozygous sample in the Barrow and SLI samples), and two had minor allele frequencies ≤ 5%. The rest have minor allele frequencies between 10% and 48% (Table 1).

We genotyped 129 samples with 19 SNP assays, producing 3321 genotypes including initial replicates, but not including repeated genotyping of the low-concentration samples. Comparing PCR success rates between modern and historical samples for all nonreplicate sample amplifications ($N = 1641$), modern samples from Barrow and SLI ($N = 86$) failed to amplify, or amplified but could not be designated a genotype, 1.5% (25/1641) of the time. Historical samples ($N = 41$ after two were removed because of consistent failure) failed to amplify or be assigned a genotype 5.7% (64/1126) of the time. If historical samples are further divided by DNA extract concentration, the 'low concentration' group (≤ 10 copies/μL) had a failure rate of 11.8% (54/456), and the 'high concentration' group had a failure rate of 1.5% (10/670). Failure rates for individual samples for which we only genotyped one extract indicate that a value of 10 copies/μL is a reasonable cut-off for selecting samples for replication, though it is possible that allelic dropout will still occur in samples with higher DNA concentrations (Fig. 4). As our qPCR assay was detecting a product of 51 bp, and the number of copies may decrease by a factor of 10 with a doubling of length in degraded samples (Poinar *et al*. 2006), it is possible that samples with < 10 copies/μL detected by qPCR have ≤ 1 copy/μL (≤ 5 copies/reaction) available for multiplex PCR of each SNP locus.

Assays varied in overall quality, but genotype clusters could be discerned with high confidence for all assays, with no qualitative or quantitative difference between the results from modern and historical samples (Fig. 5).

Not including NTCs, we replicated 26.6% of the genotypes (884/3321) prior to replicating low-concentration samples. This includes samples that were re-genotyped from the same multiplex preamplification product to determine Amplifluor genotyping error rates from the high-concentration multiplex PCR products ($N = 488$ genotypes from 85 samples), samples that were extracted twice and both extracts genotyped ($N = 366$ genotypes), and extracts that were amplified in two separate multiplex PCRs and genotyped from those

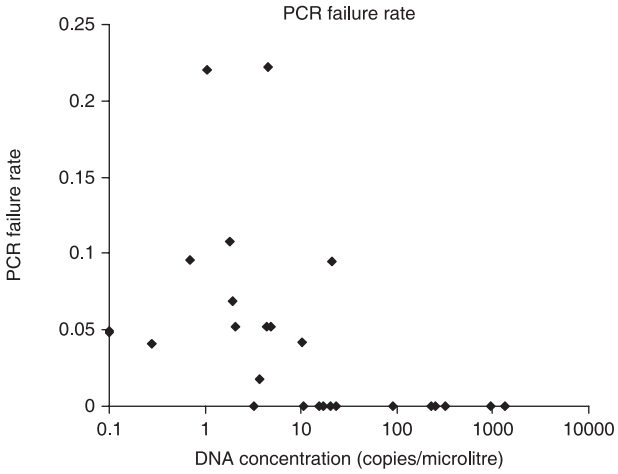

Fig. 4 Plot of the DNA concentration (copies/μL) vs. PCR failure rate for SNP genotyping. Two samples with no detectable DNA in the qPCR assay were given concentration values of 0.1 to allow them to be plotted on the logarithmic scale. The number of PCRs attempted ranged from 19 to 73 per sample, as samples with > 10 copies/μL were only genotyped once, and those with ≤ 10 copies/μL were genotyped multiple times. Each multiplex PCR contained 5 μL of sample, so the starting copy number was five times that of the concentration shown.

products (N = 30 genotypes), to determine error rates from multiplex PCR of genomic DNA templates. After excluding three samples that failed to amplify most of the time, there were only eight mismatched genotypes detected (e.g. a homozygous and a heterozygous genotype from the same template). All of these were among the bone and baleen samples, and differences were between extracts, and as expected, not between genotypes from the same multiplex PCR. This represents a genotyping error rate (skewed towards very-low-concentration samples) of eight out of 884 (0.9%) over all samples and loci. For the 488 replicate genotypes from the same multiplex PCR templates, there were no mismatched genotypes. These two error rates therefore represent the errors associated with multiplex PCR from genomic DNA (0.9%) and Amplifluor genotyping from multiplex PCR products (undetected).

Analysis of the additional replications from new multiplex PCRs from the lowest concentration samples showed a higher rate of allelic dropout and PCR failure for samples with ≤ 10 copies/μL, with 38 of 44 mismatched replicates (2371 total duplicate genotypes, all samples included) being between replicates of samples with ≤ 10 copies/μL, and three of the remaining mismatches being between genotypes from two extracts of the same sample where one extract had ≤ 10 copies/μL and the other had > 10 copies/μL. Three modern samples presumed to have > 10 copies/μL had mismatches. This resulted in an overall mismatch (error) rate of 1.9%, but only 0.1% mismatches among modern samples and historical samples with > 10 copies/μL DNA. Assuming that all mismatches were the result of allelic dropout rather than other genotyping errors (expected to be 0.1%), the 1.9% error rate reflects the rate of allelic dropout for samples with ≤ 10 copies/μL DNA. The actual allelic dropout rate may be higher, as three to four replicates were not sufficient in 14 cases to verify genotypes with the minimum number of replicates needed (see methods), and in rare cases allelic dropout still may not be detected without further genotyping.

All negative controls (159 negative extraction controls, 436 multiplex PCR NTCs, and 294 amplifluor assay NTCs), totalling 889 of 5090, or 17.5% of all PCRs, were unamplified at the cycle where genotypes were called. Subsequent fluorescent signal in some NTCs was seen in later cycles, and was most often seen in all NTCs for the given assay, indicating background noise rather than low-level cross-contamination. This is a typical assay-dependent limitation of Amplifluor SNP assays (Chemicon, pers. comm.), but is greatly reduced when genotyping from multiplex PCR products compared to genotyping from genomic DNA (data not shown).

Discussion

We have combined highly multiplexed PCR of small (< 120 bp) products, a method previously developed for sequencing of mtDNA and single copy nuclear genes from ancient DNA samples (Krause *et al*. 2006; Römpler *et al*. 2006a, b), with secondary genotyping of individual SNP assays to create a highly accurate and reproducible method for obtaining nuclear genetic data from historical and low-quality samples. Such samples have been notoriously difficult for microsatellite genotyping (e.g. Navidi *et al*. 1992; Taberlet *et al*. 1996; Morin *et al*. 2001; Wandeler *et al*. 2003), and the potential of SNPs to open up the use of poor-quality samples for assaying nuclear variation has been discussed with hopeful enthusiasm (Morin *et al*. 2004; Wayne & Morin 2004; Poinar *et al*. 2006; Römpler *et al*. 2006a). Our results indicate that that potential can be realized relatively easily when samples contain DNA concentrations > 10 copies/μL of a small single copy nuclear DNA fragment, and that even lower concentration samples can be assayed with appropriate replication to control for allelic dropout. The use of highly multiplexed preamplification allows genotyping and replication to be completed for a large number of loci without threat of exhausting the extracted DNA, as all loci can potentially be genotyped from one multiplex PCR, and subsequent replications can be completed from additional multiplex PCRs. Since the maximum number of replicates recommended is 7 (Taberlet *et al*. 1996), a sample can be quantified by qPCR (Morin *et al*. 2001, 2007b), and replication numbers determined prior to the beginning of the genotyping process to maximize efficiency while ensuring data quality.

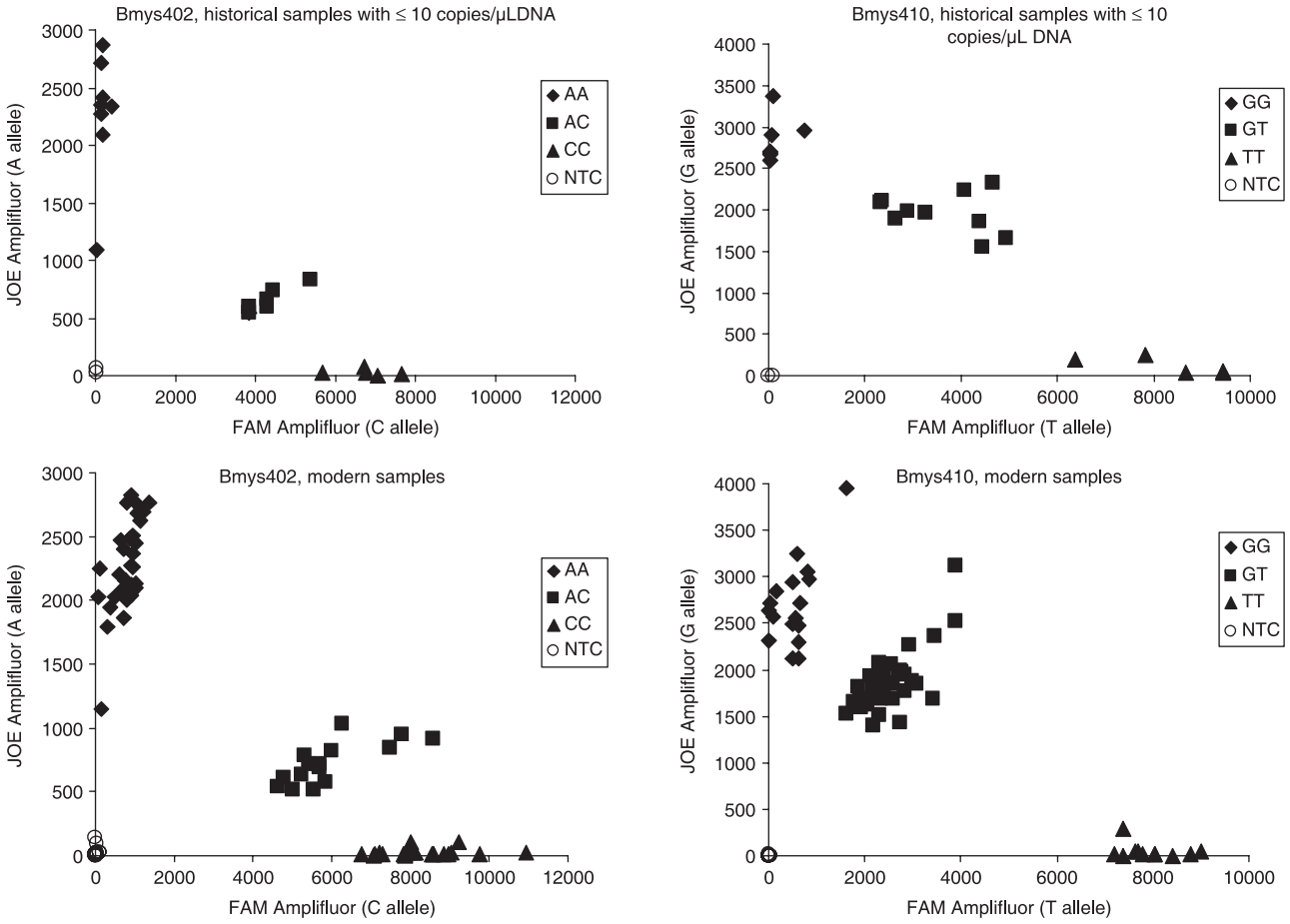

Fig. 5 Plots of Amplifluor fluorescence values for each allele-specific amplifluor for two assays, separated into modern samples and historical samples with ≤ 10 copies/μL DNA. Assay names were shortened from Bmys402M56 and Bmys410K107 to Bmys402 and Bmys410, respectively.

Although we only report here on data from a 20-locus multiplex reaction, we have subsequently optimized a 26-plex reaction including these and six additional loci. Multiplex PCR of up to 40 loci using these methods has been optimized for sequencing mtDNA from ancient cave bear samples (M. Hofreiter, pers. comm.). We have not attempted to amplify products > 130 bp because of our focus on highly degraded samples, but there is no reason that we know of that larger PCR products could not be amplified in the multiplex reactions to allow genotyping of multiple SNPs from each locus product, as long as the genomic DNA templates are not degraded.

As with all poor-quality, historical, and ancient DNA samples, contamination is a significant concern. Appropriate steps need to be taken from the outset of a study to ensure that samples do not become contaminated in any part of the process, and to monitor for evidence of contamination. Our study has shown that, with proper care (e.g. separate sample handling and extraction facilities, preparation of dry plates to minimize sample handling) and use of extraction, multiplex, and PCR negative controls, genotypes can be generated with little doubt of their accuracy. Indeed, error rates for SNP genotyping from historical samples were 0.1% for all but the poorest quality samples, lower than is often reported for microsatellite genotypes obtained from modern samples (e.g. 0.8% from tissue, 2.0% from faeces; Bonin *et al*. 2004). The primary concern remains that low-concentration DNA can result in high levels of allelic dropout. Apparently high-quality genotypes (e.g. high signal strength and clear genotype clusters) are no assurance that allelic dropout is not occurring, but frequent PCR failure is a strong predictor of allelic dropout, so DNA quantification and/or appropriate levels of replication are necessary to ensure accurate genotypes. Without prior evaluation of the error rates associated with different starting template quantities, it has been recommended that low-quality samples be replicated up to seven times to maximize the probability of detecting both alleles (Taberlet *et al*. 1996).

DNA quantification to evaluate samples and allelic dropout rates prior to beginning a genotyping project can significantly reduce the number of replicates needed (Morin *et al*. 2001).

Multiplex PCR offers an efficient way to generate high-quality genotype data, but does not get around the need to prescreen samples and replicate low-concentration samples to verify genotypes. Combination of multiplex PCR with Amplifluors makes optimization easier and produces higher quality data than Amplifluors alone at a low per-genotype cost, especially with reduced amplifluor primer concentrations. We have estimated that our multiplex PCR cost is $US0.65 per reaction, and our per genotype cost is approximately $US0.19 (not including multiplex PCR, assay optimization and replication). The total cost will depend on the number of multiplex PCR replicates to be performed for each sample, and the number of SNP assays per multiplex PCR. As an example, multiplex PCR of 20 assays, replicated three times, would result in a total cost of $US13.35 per completed SNP genotype profile (all 20 loci), or $US0.67 per completed genotype. As genotyping methods are constantly evolving, and different laboratories will choose different methods based on their throughput needs and available equipment, we expect the cost of genotyping to be lower in the future, but variable among laboratories. The method we have developed serves several purposes, minimizing costs while allowing for reasonably high-throughput genotyping. Additional modifications will be needed to further decrease costs, increase throughput to allow for efficient amplification of a larger number of loci in the multiplex PCR (e.g. Shapero *et al*. 2004), and facilitate higher throughput genotyping (e.g. using a multiplex SNP assay format).

Acknowledgements

We are grateful to Craig George (North Slope Borough Department of Wildlife Management), Merlin Koomooka and George Noongwook (Alaska Eskimo Whaling Commission) for collection of the bowhead tissue, bone and baleen samples. Brittany Hancock and Janet Lowther provided laboratory assistance, and Bela Dornon and Henry Orr helped with figure graphics. This research was supported by the SWFSC and a grant from the North Pacific Research Board. DNA sequences from a bowhead whale genomic library were generously provided by John Bickham and Ryan Huebinger for PCR primer selection for SNP discovery. We are grateful for discussion and review of early versions of the manuscript by Michi Hofreiter, Rick LeDuc, and Matthias Meyer. All samples were collected by or transferred to the SWFSC under NMFS/MMPA and CITES permits.

References

Aitken N, Smith S, Schwarz C, Morin PA (2004) Single nucleotide polymorphism (SNP) discovery in mammals: a targeted-gene approach. *Molecular Ecology*, **13**, 1423–1431.

Asher RJ, Hofreiter M (2006) Tenrec phylogeny and the noninvasive extraction of nuclear DNA. *Systematic Biology*, **55**, 181–194.

Bonin A, Bellemain E, Bronken Eidesen P *et al*. (2004) How to track and assess genotyping errors in population genetics studies. *Molecular Ecology*, **13**, 3261–3273.

Brumfield RT, Beerli P, Nickerson DA, Edwards SV (2003) The utility of single nucleotide polymorphisms in inferences of population history. *Trends in Ecology & Evolution*, **18**, 249–255.

Chakraborty R, Stivers DN, Su B, Zhong Y, Budowle B (1999) The utility of short tandem repeat loci beyond human identification: implications for development of new DNA typing systems. *Electrophoresis*, **20**, 1682–1696.

Elfstrom CM, Smith CT, Seeb JE (2006) Thirty-two single nucleotide polymorphism markers for high-throughput genotyping of sockeye salmon. *Molecular Ecology Notes*, **6**, 1255–1259.

Glaubitz JC, Rhodes OE, Dewoody JA (2003) Prospects for inferring pairwise relationships with single nucleotide polymorphisms. *Molecular Ecology*, **12**, 1039–1047.

Hedrick PW (2005) A standardized genetic differentiation measure. *International Journal of Organic Evolution*, **59**, 1633–1638.

Kalinowski ST (2002) How many alleles per locus should be used to estimate genetic distances? *Heredity*, **88**, 62–65.

Kohn MH, Murphy WJ, Ostrander EA, Wayne RK (2006) Genomics and conservation genetics. *Trends in Ecology & Evolution*, **21**, 629–637.

Krause J, Dear PH, Pollack JL *et al*. (2006) Multiplex amplification of the mammoth mitochondrial genome and the evolution of Elephantidae. *Nature*, **439**, 724–727.

Kuhner MK, Beerli P, Yamato J, Felsenstein J (2000) Usefulness of single nucleotide polymorphism data for estimating population parameters. *Genetics*, **156**, 439–447.

Lindblad-Toh K, Winchester E, Daly MJ *et al*. (2000) Large-scale discovery and genotyping of single-nucleotide polymorphisms in the mouse. *Nature Genetics*, **24**, 381–386.

Markoulatos P, Siafakas N, Moncany M (2002) Multiplex polymerase chain reaction: a practical approach. *Journal of Clinical Laboratory Analysis*, **16**, 47–51.

Markoulatos P, Siafakas N, Katsorchis T, Moncany M (2003) Multiplex PCR: rapid DNA cycling in a conventional thermal cycler. *Journal of Clinical Laboratory Analysis*, **17**, 108–112.

Morin PA, Saiz R, Monjazeb A (1999) High throughput single nucleotide polymorphism genotyping by the 5′ exonuclease assay. *BioTechniques*, **27**, 538–552.

Morin PA, Chambers KE, Boesch C, Vigilant L (2001) Quantitative polymerase chain reaction analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). *Molecular Ecology*, **10**, 1835–1844.

Morin PA, Luikart G, Wayne RK, SNP-Workshop Group (2004) SNPs in ecology, evolution and conservation. *Trends in Ecology & Evolution*, **19**, 208–216.

Morin PA, LeDuc RG, Robertson KM *et al*. (2006) Genetic analysis of killer whale (*Orcinus orca*) historical bone and tooth samples to identify western U.S. ecotypes. *Marine Mammal Science*, **22**, 897–909.

Morin PA, Aitken NC, Rubio-Cisneros N, Dizon AE, Mesnick SL (2007a) Characterization of 18 SNP markers for sperm whale (*Physeter macrocephalus*). *Molecular Ecology Notes*, in press, online. doi: 10.1111/j.1471–8286.2006.01654.x.

Morin PA, Hedrick NM, Robertson KM, LeDuc CA (2007b) Comparative mitochondrial and nuclear quantitative PCR of historical marine mammal tissue, bone, baleen, and tooth samples. *Molecular Ecology Notes*, in press, online. doi: 10.1111/j.1471–8286.2007.01699.x.

Navidi W, Arnheim N, Waterman MS (1992) A multiple-tubes approach for accurate genotyping of very small DNA samples by using PCR: statistical considerations. *American Journal of Human Genetics*, **50**, 347–359.

Nielsen R (2000) Estimation of population parameters and recombination rates from single nucleotide polymorphisms. *Genetics*, **154**, 931–942.

Noonan JP, Hofreiter M, Smith D *et al*. (2005) Genomic sequencing of Pleistocene cave bears. *Science*, **309**, 597–599.

Pääbo S, Poinar H, Serre D *et al*. (2004) Genetic analyses from ancient DNA. *Annual Review of Genetics*, **38**, 645–679.

Poinar HN, Schwarz C, Qi J *et al*. (2006) Metagenomics to paleogenomics: large-scale sequencing of mammoth DNA. *Science*, **311**, 392–394.

Primmer CR, Borge T, Lindell J, Saetre GP (2002) Single-nucleotide polymorphism characterization in species with limited available sequence information: high nucleotide diversity revealed in the avian genome. *Molecular Ecology*, **11**, 603–612.

Römpler H, Dear PH, Krause J *et al*. (2006a) Multiplex amplification of ancient DNA. *Nature Protocols*, **1**, 720–728.

Römpler H, Rohland N, Lalueza-Fox C *et al*. (2006b) Nuclear gene indicates coat-color polymorphism in mammoths. *Science*, **313**, 62.

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds S Krawetz, S Misener), pp. 365–386. Humana Press, Totowa, New Jersey.

Ryman N (2006) CHIFISH: a computer program testing for genetic heterogeneity at multiple loci using chi-square and Fisher's exact test. *Molecular Ecology Notes*, **6**, 285–287.

Ryman N, Palm S (2006) POWSIM: a computer program for assessing statistical power when testing for genetic differentiation. *Molecular Ecology Notes*, **6**, 600–602.

Ryman N, Palm S, Andre C *et al*. (2006) Power for detecting genetic divergence: differences between statistical methods and marker loci. *Molecular Ecology*, **15**, 2031–2045.

Seddon JM, Parker HG, Ostrander EA, Ellegren H (2005) SNPs in ecological and conservation studies: a test in the Scandinavian wolf population. *Molecular Ecology*, **14**, 503–511.

Shapero MH, Zhang J, Loraine A *et al*. (2004) MARA: a novel approach for highly multiplexed locus-specific SNP genotyping using high-density DNA oligonucleotide arrays. *Nucleic Acids Research* 32, e181.

Smith S, Aitken N, Schwarz C, Morin PA (2004) Characterisation of 15 single nucleotide polymorphism markers for chimpanzees (*Pan troglodytes*). *Molecular Ecology Notes*, **4**, 348–351.

Surridge AK, Smith AC, Buchanan-Smith HM, Mundy NI (2002) Single-copy nuclear DNA sequences obtained from noninvasively collected primate feces. *American Journal of Primatology*, **56**, 185–190.

Taberlet P, Griffin S, Goossens B *et al*. (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, **24**, 3189–3194.

Wandeler P, Morin PA, Smith S, Pettifor RA, Funk SM (2003) Patterns of nuclear DNA degeneration over time — a case study in historic teeth samples. *Molecular Ecology*, **12**, 1087–1093.

Wayne RK, Morin PA (2004) Conservation genetics in the new molecular age. *Frontiers in Ecology and the Environment*, **2**, 89–97.